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UV Disinfection of Adenoviruses: Molecular Indications of DNA Damage Efficiency▽

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Adenovirus is a focus of the water treatment community because of its resistance to standard, monochromatic low-pressure (LP) UV irradiation. Recent research has shown that polychromatic, medium-pressure (MP) UV sources are more effective than LP UV for disinfection of adenovirus when viral inactivation is measured using cell culture infectivity assays; however, UV-induced DNA damage may be repaired during cell culture infectivity assays, and this confounds interpretation of these results. Objectives of this work were to study adenoviral response to both LP and MP UV using (i) standard cell culture infectivity assays and (ii) a PCR assay to directly assess damage to the adenoviral genome without introducing the virus into cell culture. LP and MP UV dose response curves were determined for (i) log inactivation of the virus in cell culture and (ii) UV-induced lesions per kilobase of viral DNA as measured by the PCR assay. Results show that LP and MP UV are equally effective at damaging the genome; MP UV is more effective at inactivating adenovirus in cell culture. This work suggests that the higher disinfection efficacy of MP UV cannot be attributed to a difference in DNA damage induction. These results enhance our understanding of the fundamental mechanisms of UV disinfection of viruses—especially double-stranded DNA viruses that infect humans—and improve the ability of the water treatment community to protect public health.

Adenoviruses are human pathogens that can be transmitted via the fecal-oral and respiratory routes (25, 34, 42, 43). There are 52 serotypes that infect humans, causing primarily eye and respiratory infections and potentially severe enteric dysentery (34, 42). The enteric adenoviruses, types 40 and 41, have been studied the most by the water treatment community, but research on these serotypes is somewhat limited by the difficulties associated with propagating them in vitro (5, 23). Adenovirus types 2 and 5 are used more often in laboratory research because they can be grown to higher titer and more is known about them (32). All of the adenoviruses are nonenveloped, icosahedral particles consisting of a protein coat, or capsid, surrounding a DNA-protein core; they range in size from 70 to 100 nm (34). The adenoviral genome is double-stranded DNA like that of its mammalian hosts and varies in length from approximately 30 to 40 kb, depending on the serotype. In addition to those in the coat, there are proteins in the viral core that are closely associated with the DNA. The viral proteins are integral to the infection process, and an adenovirus with damaged DNA can successfully infect host cells (32). Thus, optimal disinfection of adenovirus requires damage to more than just the viral DNA.

All serotypes of adenovirus studied to date have shown significant resistance to standard UV disinfection compared with other waterborne viruses, including echoviruses, coxsackieviruses, rotaviruses, and caliciviruses (2, 10, 15, 16, 19, 20, 22, 26, 33, 36). The response of adenoviruses to UV disinfection is of such concern that a special workshop was convened on the science of adenoviruses and the impact of their apparent UV resistance on UV disinfection requirements for water treatment (43). Adenovirus' UV resistance has had a significant impact on regulations: in the Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR), the U.S. Environmental Protection Agency (USEPA) established that a delivered UV dose of 186 mJ/cm^2 is required for 4-log inactivation of all viruses; prior to the promulgation of the LT2ESWTR in January 2006, a UV dose of 40 mJ/cm^2 was considered sufficient (37, 39). The Groundwater Rule was promulgated in January 2007 and states that UV is not sufficient as a stand-alone treatment for 4-log inactivation of any viruses (40). Both of these rules are based on adenoviruses, which are currently thought to be the most UV-resistant class of viruses and are therefore used as a standard for viral inactivation requirements. Until recently, adenovirus was consistently listed on the USEPA's Contaminant Candidate List, which names high-priority targets for research and data collection (38). Recent cases of serious and sometimes fatal pneumonia caused by adenovirus type 14 (9) serve as a reminder of the threat adenoviruses pose to public health, since there is no cure or highly effective therapy for them.

A significant amount of data has been published on UV inactivation of adenovirus and other viruses using monochromatic low-pressure (LP) UV irradiation, followed by assays of infectivity using cell culture (2, 4, 5, 15, 16, 19, 20, 24, 26, 33, 35, 36). These studies have shown that 4-log inactivation of adenovirus requires a LP UV dose of up to 200 mJ/cm^2, while 30 to 40 mJ/cm^2 is sufficient to cause 4-log inactivation of other viruses (15, 24, 33). It is possible that the higher dose requirement for LP UV inactivation of adenovirus reflects not true resistance but rather repair of damaged adenoviral DNA in host cells during the cell culture infectivity assays. LP UV used

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TABLE 1. UV irradiation conditions

UV lamp and expt	Avg irradiance (mW/cm^2)	Exposure time (s) for 50-mJ/cm^2 dose	UV absorbance at 254 nm
LP			
1	0.3935	127	0.33
2	0.3602	139	0.20
3	0.3617	138	0.18
MP			
1	0.4415	113	0.33
2	0.4291	116	0.20
3	0.4500	111	0.18

in the studies described above is nearly monochromatic at 253.7 nm—very near the 260 nm absorbance maximum of nucleic acids, such as DNA and RNA, that make up the genomes of viruses and other pathogens. It is widely accepted that LP UV inactivates microorganisms by damaging their genomes. Because adenovirus can infect host cells even when its genome is damaged (32) and because that genome is double-stranded DNA like the genome of the host cell, it follows that the DNA repair machinery of the host cell might recognize and repair damage to the adenoviral genome during standard cell culture infectivity assays. Similar effects in cell culture have likely not been seen in other waterborne viruses because their genomes are single-stranded or composed of RNA (4, 10, 15, 16, 24, 29, 36) and are therefore not recognized by host cell DNA repair machinery. Furthermore, when irradiated with medium-pressure (MP) UV, adenoviruses have been shown to be as susceptible to UV inactivation as other viruses, even in standard cell culture infectivity assays (22). MP UV is polychromatic—it emits a range of wavelengths, including those which are absorbed by proteins, so it has the potential to damage the viral coat and core proteins in addition to the genome. Such extragenomic damage appears to play an important role in viral inactivation.

This study was designed to help clarify the effects of UV on adenovirus using a PCR assay that is carried out directly on irradiated adenoviruses without cell culture, thus eliminating the confounding effects of possible DNA repair in cell culture. In the work described here, we treat adenovirus using both LP and MP UV, and we assess the UV-treated viruses using both standard cell culture infectivity assays and a PCR assay for DNA damage. This PCR assay is designed not for detection or quantitation of the virus itself but for quantitation of damage to the viral genome; it allows direct assessment of damage to the adenoviral DNA after irradiation without introducing the virus into host cells. This is the first report we know of in which UV-induced DNA damage to adenovirus has been examined directly, without the confounding effects of a host cell system. Taken together, the cell culture and PCR data help clarify the effects of both LP and MP UV on damage to the adenoviral genome as well as the role this damage plays in viral inactivation.

MATERIALS AND METHODS

UV irradiation and experimental design. Both LP and MP UV lamps were housed in separate bench scale "collimated beam" apparatuses. The UV dose was calculated as the average irradiance of the wavelengths in the completely mixed batch irradiation vessel multiplied by the time of exposure as described by Bolton and Linden (6). For the MP UV source, irradiance was weighted using the DNA absorbance spectrum to provide a "germicidal" dose of between 200 and 300 nm. The absorbance spectrum of each sample across the entire germicidal range was accounted for in the dose calculations, and a petri factor was applied in all cases to account for variation in UV intensity across the surface of the petri dish. Such variation in intensity was minimal: the petri factors for LP and MP UV were, respectively, 0.98 and 1.00. Average UV irradiance, exposure times for the 50 mJ/cm^2 dose, and UV absorbance at 254 nm are listed for each experiment in Table 1. Five milliliters of viral suspension in phosphate-buffered saline (PBS) was irradiated for each sample; the sample depth in the petri dish was 0.23 cm for all samples, and all samples were stirred constantly during irradiation. The relative lamp emission as a function of wavelength for LP UV and MP UV is shown in Fig. 1. Three independent UV irradiation experiments were performed for this work, and each sample was split into two parts: one part was used for cell culture infectivity assays, and the other was used for PCR. One set of cell culture infectivity data and two sets of PCR data were obtained for each independent UV irradiation experiment.

Culture of stock cells, preparation of virus, and infectivity assays. The A549 cell line (American Type Culture Collection, Manassas, VA) was used to propagate the virus and was used for all cell culture infectivity assays. The cell line was carried in Ham's F-12K medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Replating of stock cells was done by PBS rinsing, trypsinization with 0.25% trypsin/EDTA, resuspension, and dilution into new flasks. Cell stocks were split once per week, and medium was changed twice per week. Media and solutions for cell culture and viral propagation were obtained from Invitrogen (Carlsbad, CA).

Adenovirus type 2 was a gift from Gwy-Am Shin (University of Washington, Seattle, WA); the virus was propagated in host cells and then concentrated and purified via polyethylene glycol precipitation as previously described (36). Briefly, A549 host cell monolayers in 150-cm^2 flasks were inoculated with 1 ml of Dulbecco's PBS (Invitrogen, Carlsbad, CA) containing 10^6 most-probable number per milliliter of virus. MPN is explained below in more detail. Viruses were allowed to adsorb to host cells for 1 to 1.5 h at 37°C and were rocked gently by hand once every 15 min before adding 60 ml of viral propagation medium per flask (high-glucose Dulbecco's modified Eagle's medium [DMEM], 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B). Flasks were incubated at 37°C for 9 to 10 days to allow viral replication and then frozen and thawed twice to break open host cells. The resulting solution was centrifuged at 6,000 × *g* for 15 min at 4°C to remove cellular debris, and supernatant containing viruses was stirred with polyethylene glycol, at a molecular weight of 8,000 (9 g/100 ml supernatant), and NaCl (5.8 g/100 ml supernatant) for 2.5 h at room temperature. Viruses were then pelleted by centrifugation at 6,000 × *g* for 30 min at 4°C, resuspended in PBS, and extracted once with chloroform to disperse the virus. Viral stocks were kept at 4°C for short-term storage or at −80°C for long-term storage.

Cell culture infectivity assays were used both to determine the titer of viral stocks and to assay UV-irradiated virus. A549 cells were plated into 25-cm^2 flasks at a density of 3×10^5 to 3.5×10^5 cells per flask in complete DMEM (high-glucose DMEM, 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and either 0.25 or 2.5 μg/ml amphotericin B) and allowed to grow for 2 days at 37°C and 5% CO_2. At least three different dilutions of virus and at least three different flasks per dilution were inoculated onto cells; cells and viruses were incubated at 37°C in a 5% CO_2 incubator for up to 3 weeks before being scored. Flasks were scored as positive or negative for cytopathic

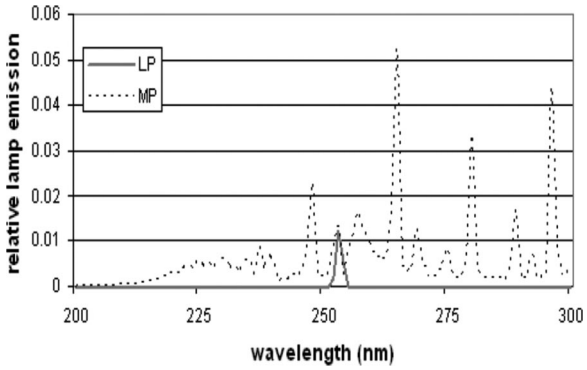

FIG. 1. LP and MP mercury vapor UV lamp emission spectra.

TABLE 2. Adenovirus type 2 primers

Left sequence	Right sequence	Genome region	Product size (bp), source
CCCGTTTTCGCTCGTCACATCC	CGCCCGACTTGTTCCTCGTTTG	23162–24352	1,190
CGGTTTCCTGTCGAGCCAAACG	CCCGCACCTGGTTTTGCTTCAG	33045–34104	1,059
CAGGAATCGCCCCATCATCGTC	CGCCCGACTTGTTCCTCGTTTG	23336–24352	1,016, this study
CCGCCGTGAACCCCGAGTATTT	TCGTTACCCTCGGGCACCTCAA	13428–13530	102
GACAGCGTGTTTTCCCCGCAAC	TGGCCTGCGGAAGCTTTCCTTT	13556–13651	95
CCGCCGTGAACCCCGAGTATTT	TGGCCTGCGGAAGCTTTCCTTT	13428–13651	223

effects, and scoring data were entered into a computer program which calculates MPN per milliliter as described by Hurley and Roscoe (17). Concentration of amphotericin B did not appear to affect the number of positive and negative flasks in cell culture infectivity assays (data not shown). Log inactivation for UV irradiation studies was calculated as $\log_{10}$[(MPN/ml untreated control)/(MPN/ml UV-treated sample)], and log survival was calculated as −(log inactivation).

PCR assay for DNA damage. (i) Background. The PCR assays developed for use on adenovirus so far have involved the amplification of short stretches of DNA and are not designed to help one assess the structural integrity of the genome as a whole (12, 18, 19, 20, 21). Amplification of long stretches of DNA combined with PCR is powerful with respect to both its sensitivity and its target specificity in detection of DNA damage, and this method has been extensively investigated by the van Houten group, which refers to it as the "quantitative PCR assay for DNA damage" (1, 11, 31, 41; see also reference 3). The use of PCR to determine DNA damage levels is based on the fact that progression of the polymerases used to amplify DNA in PCRs is inhibited by DNA damage. Equal amounts of DNA from samples containing different amounts of DNA damage therefore differ in the extent to which they can be amplified; samples with less damage undergo greater amplification. When amplification is stopped while the PCR is still in the exponential phase, the yield is directly proportional to the damage level and the amount of PCR product can be used for accurate quantitation of DNA damage. This assay is not conducted using real-time PCR; rather, the range of cycle numbers in which amplification is exponential is determined using cycle tests which are carried out on DNA from untreated virus prior to PCR on experimental samples. Subsequent PCR on experimental samples is carried out using a standard Thermocycler rather than a real-time PCR machine; PCR is stopped after the number of cycles is identified as producing quantitative results in the preliminary cycle tests (41). Early optimization tests also demonstrated the selective amplification of a single PCR band of the expected size, as detected on ethidium bromide-stained agarose gels (data not shown).

(ii) Method. DNA was extracted from 200 μl of irradiated adenovirus using the QIAamp DNA blood mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Concentration of this viral template DNA (in nanograms/microliter) was determined using PicoGreen from Molecular Probes (Invitrogen, Carlsbad, CA) in a 96-well microplate according to the manufacturer's instructions. All PCRs were set up using 0.5 ng of adenoviral template DNA in a total of 10 μl buffer AE, supplied with the DNA extraction kit (Qiagen, Valencia, CA). Primers for adenovirus type 2 were designed specifically for this work. The primers used here result in a 1,016-bp product that spans the genome from bp 23336 to 24352. We designed and tested six pairs of primers specific for adenovirus type 2 that worked well for our PCR conditions and gave a range of product sizes. Table 2 shows sequences, product sizes, and location in the adenoviral genome for all six of these primer pairs, including those used for this study.

PCR was performed using the GeneAmp XL PCR kit (Applied Biosystems, Foster City, CA); final concentrations in 50 μl total reaction volume were as follows: 1× PCR buffer, 800 μM deoxynucleoside triphosphates (200 μM of each deoxynucleoside triphosphate), 1.2 mM magnesium acetate, and 1 U r*Tth* DNA polymerase, all supplied with the kit; we added bovine serum albumin to a final concentration of 0.1 mg/ml and added left and right primers to a final concentration of 0.40 μM each, and each reaction mixture had 0.5 ng of template DNA. PCRs were run in a Biometra Thermocycler (Gottingen, Germany). Thermal conditions for the PCR were as follows: 72°C for 2 min, 94°C for 1 min, 94°C for 15 s (first step of cycle), 65°C for 3 min (second step of cycle), 72°C for 5 min, and holding at 8°C. Enzyme was added to each reaction after 1.5 min at 72°C. Eighteen to 19 cycles were performed for all PCRs except those indicated above, and this number was determined using cycle tests as described above (41). PCR products were quantified using PicoGreen in the same manner as the template DNA, and amplification of UV irradiated samples relative to an untreated (undamaged) control—relative amplification—was determined using a simple ratio of the quantity of DNA in the treated sample to the quantity of DNA in the untreated control (31). UV-induced lesions per kilobase of viral DNA were calculated using the negative log of the relative amplification according to Ayala-Torres et al. (1).

Statistical analysis. Cell culture infectivity and lesion data were analyzed by two-factor analysis of variance (ANOVA), and post hoc analyses were carried out where appropriate using Fisher's protected least significant differences test. Statistical analyses were carried out using StatView for Windows (version 5.0.1; SAS Institute, Inc., Cary, NC).

RESULTS

Cell culture infectivity assays. Results of the cell culture infectivity assays are shown in Fig. 2, with UV dose on the *x* axis and log survival on the *y* axis. Points on the plot are an average of the data from three independent experiments, one set of data per experiment; error bars show one standard error of the mean above and below the average. These data show that the UV dose requirement for a given level of inactivation using LP UV is consistently higher than the dose required for the same level of inactivation using MP UV. ANOVA shows highly significant main effects for both lamp type ($P < 0.0001$) and UV dose ($P < 0.0001$) as well as a highly significant interaction for lamp · dose ($P = 0.0004$). Pairwise post hoc comparisons (Fisher's protected least significant differences) indicate that the difference in inactivation between LP and MP UV is highly significant at 25 mJ/cm^2 ($P = 0.005$) and 50 mJ/cm^2 ($P = 0.0003$); using a *P* value of 0.05 as a cutoff, differences were not significant at 10 mJ/cm^2 ($P = 0.08$) or 125 mJ/cm^2 ($P = 0.37$). Four-log inactivation requires an LP UV

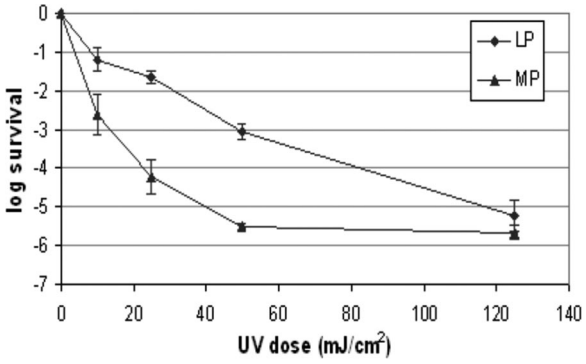

FIG. 2. UV inactivation of adenovirus as determined by cell culture infectivity assay data.

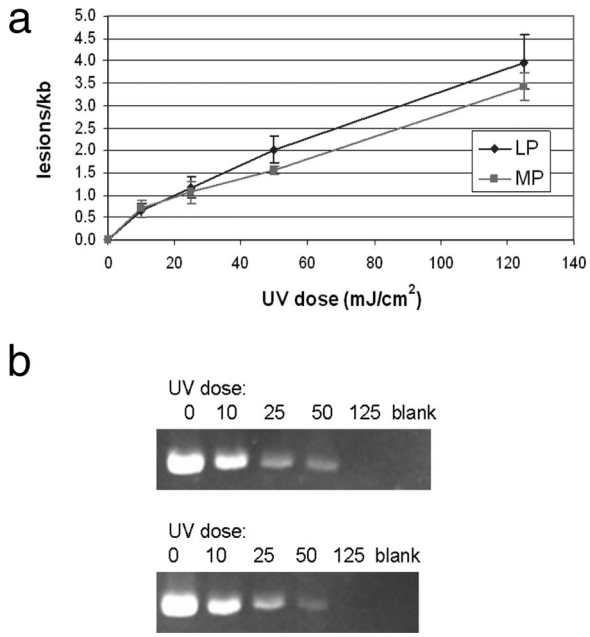

FIG. 3. PCR assay for DNA damage showing relative amplification data. (a) Lesions per kilobase of viral DNA. (b) Agarose gel showing PCR products using LP UV (top) and MP UV (bottom). Bands were visualized on a 1% agarose gel run with sodium borate buffer and stained with ethidium bromide (8).

dose of approximately 80 mJ/cm^2 and only 25 mJ/cm^2 of MP UV.

PCR: DNA damage of UV-treated adenovirus. Figure 3a shows lesions per kilobase of adenoviral DNA as a function of UV dose for both LP and MP UV—these data are taken from viruses that were exposed to UV but not introduced into cell culture. Lesions induced per kilobase of viral DNA were calculated as follows: negative log(relative amplification)/amplicon size (1). Two PCR replicates were run for each of the three independent experiments; lesions per kilobase were calculated for each PCR replicate, and these two replicates were averaged to determine lesions per kilobase for each of the three independent experiments. The mean and standard error of these three averages for each UV dose are shown in Fig. 3a. The figure shows an increase in lesions per kilobase with increasing UV doses. Figure 3b shows an image of the bands obtained when representative PCR products were run on a gel; as UV dose increases, the bands get narrower and fainter because there are greater DNA damage and fewer PCR products. Again, the results are very similar for both LP and MP UV. ANOVA on the lesion data indicates that the main effect for UV dose is highly significant ($P < 0.0001$), while the main effect for lamp is barely significant ($P = 0.045$). The ANOVA interaction term for dose · lamp was not significant ($P = 0.24$), so pairwise post hoc comparisons for each dose were not conducted.

DISCUSSION

Cell culture infectivity assays. Based on the data presented in Fig. 2, 2-, 3-, and 4-log inactivation of adenovirus type 2 can be achieved using LP UV doses of approximately 30, 50, and 80 mJ/cm^2, respectively. With MP UV, 2.5- and 4.5-log reductions can be reached with only 10 and 25 mJ/cm^2, and an MP UV dose of 50 mJ/cm^2 yields >5-log reduction in viral infectivity. Some previous studies have shown adenovirus to be more resistant to LP UV than the current one, requiring >100 mJ/cm^2 for 3-log inactivation and from 120 to 200 mJ/cm^2 for 4-log inactivation (5, 15, 22, 24, 26, 36). Factors that might account for some of the differences between studies include differences in viral preparation method—specifically the number of freeze-thaw cycles—as well as storage time and temperature of the virus, cell line used for the infectivity assay, virus serotype, and water used for irradiation. Differences in adenoviral response to UV have been found for buffered, demand-free groundwater and wastewater (35, 36). Other factors that are usually not cited but may account for differences between studies are the cell line used for viral propagation and the history of the viral stock itself. It is worth noting that the data presented here are in close agreement with those of Shin et al. (33); in both cases, the virus was propagated and assayed in A549 cells, and the original viral stock used to propagate all adenovirus used in the current study was obtained from G.-A. Shin. In the studies cited above, adenovirus was propagated in PLC/PRF/5, HeLa, or HEK 293 cells. In all of these studies, however, it has been shown that all serotypes of adenovirus are more resistant to LP UV than other waterborne human viruses studied so far.

The only other study published to date using MP UV to inactivate adenovirus (22) indicates that UV doses of 10 and 25 mJ/cm^2 achieve 1- and 2-log inactivation of adenovirus type 40; here, these doses caused 2- and 4-log inactivation of adenovirus type 2. This is likely due to factors cited above or differences in the method used to calculate inactivation; Linden et al. (22) used 50% tissue culture infective dose, while the current study used MPN. In both cases, MP UV is more effective at inactivating adenovirus than LP UV. This is likely because the polychromatic MP UV is capable of causing more widespread damage to the viral particle than monochromatic LP UV, which essentially damages only the DNA. The enhanced inactivation seen with MP UV could be a direct result of such extragenomic damage, or it could be because damage to the viral proteins prevents repair of genomic damage. The dose response for MP UV inactivation of adenovirus is similar to the dose response for LP UV inactivation of other waterborne and enteric viruses, including echoviruses, coxsackieviruses, and rotavirus (10, 15). Since damage to the genome is the primary mechanism of inactivation, following LP UV treatment of these other viruses, it may also be the most important factor for MP UV inactivation of adenovirus. This could be the case if the more widespread damage caused by MP UV—e.g., to viral proteins—were significant less in its own right and more in that it prevents or interferes with repair of the damaged DNA. Ongoing work in our laboratory directly examining the viral proteins is being carried out to test this hypothesis.

PCR assay for determining DNA damage of UV-treated adenovirus. PCR technologies have been applied to studies of adenovirus; however, these studies have involved either combinations of PCR and cell culture for tests of viral infectivity after UV treatment or simple detection of adenoviral DNA in untreated environmental samples (12, 18, 19, 20, 21). Methods that combine PCR with cell culture techniques are more sen-

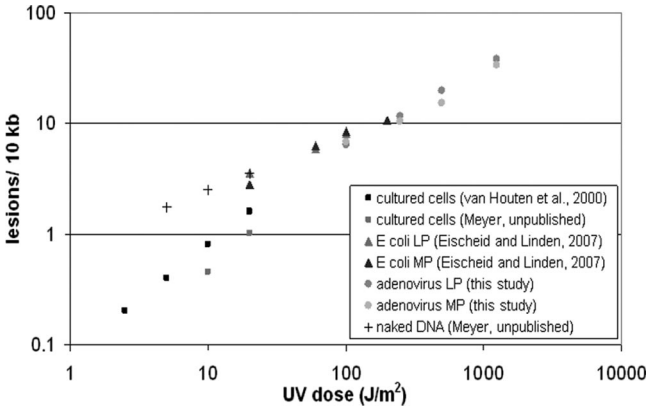

FIG. 4. UV irradiation-induced DNA lesion data from this and previous studies.

sitive than previous methods but still involve introduction of a second biological system (the cultured cells), which can obfuscate interpretation of results, and they are more time consuming than direct molecular methods might be. The PCR assay described here was used directly on irradiated adenoviruses that were exposed to UV irradiation but were not introduced into cell culture. Figure 3 shows lesions per kilobase of DNA from irradiated adenoviruses. It shows that LP and MP UV are equally effective at damaging the viral DNA at these doses, despite MP UV's improved ability to inactivate the virus in the cell culture infectivity assays. The data shown suggest that LP UV may even be slightly more efficient than MP UV at causing DNA damage; this may be because nearly all of the emission from an LP lamp targets DNA, while MP UV has emissions at other wavelengths which are not absorbed as efficiently by DNA.

Taken together, the cell culture infectivity and DNA damage data presented here support the conclusion that damage induced in adenoviral DNA by LP, 254-nm UV gets repaired in cell culture. In fact, reports from the medical literature in which 254-nm-UV-irradiated adenoviruses are assayed for infectivity in host cells that are known to be deficient in DNA repair clearly indicate that adenovirus is sensitive to UV when assayed in these cells (13, 27, 28). Boszko and Rainbow (7) have shown decreased removal of UV photoproducts from an adenoviral vector in these same repair-deficient cells—compared to normal human cells—using a quantitative PCR assay for DNA damage.

The lesion data for this study are in agreement with lesion data for previous studies: Fig. 4 shows the number of lesions (per 10 kb) introduced into adenoviral DNA in this study compared to lesions induced in cultured cells, *E. coli*, and naked DNA in other studies, using both LP and MP UV, as a function of UV dose (in J/m^2). In general, the relationship between lesions and UV dose remains linear across organisms and UV lamp type, even when different methods are used to measure the DNA damage; Eischeid and Linden (14) used an enzyme-sensitive site assay, while the other studies used a PCR assay like the one described in this paper (41).

Conclusions. This is the first study we know of in which the effect of UV irradiation on adenoviral DNA has been directly examined at the molecular level without introducing the virus into cell culture and only the second peer-reviewed paper documenting the response of adenovirus to polychromatic MP UV. We have shown that LP UV is efficient at damaging the viral DNA, and our work supports the hypothesis that this damage is repaired in cell culture. The PCR assay applied here has enhanced our understanding of the fundamental aspects of UV disinfection of adenovirus and can be adapted for use with other pathogens.

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